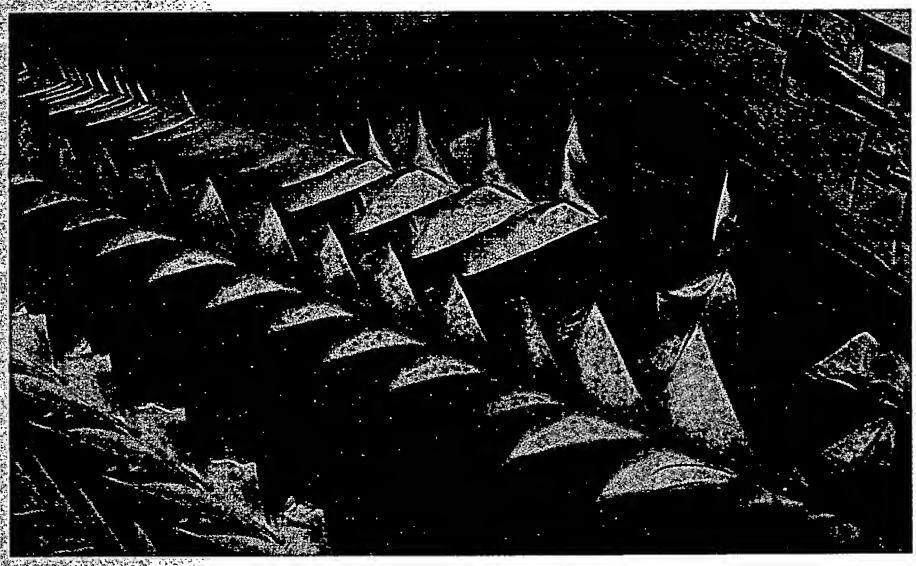
Exhibit A

Concepts IN *Biochemistry*



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8.4 Polysaccharides

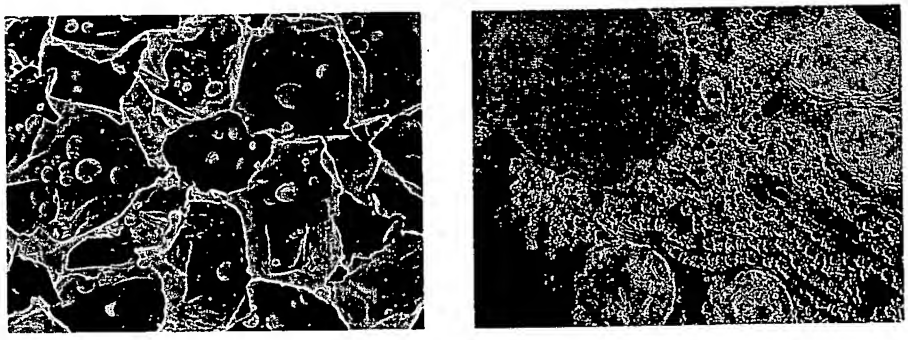
Serving as monomeric units, the monosaccharides and their derivatives are linked together to form a wide variety of polysaccharides that play diverse biological roles. The stability and variety of *O*-glycosidic bonds make it possible for monosaccharides to combine into structurally distinct and biologically useful polymers. To define the structure of a polysaccharide, several structural features must be recognized:

1. The identity of the monomeric units.
2. The sequence of monosaccharide residues (if more than one kind is present).
3. The types of glycosidic bonds linking the units.
4. The approximate length of the chain (the approximate number of monosaccharide units).
5. The degree of branching.

Homopolysaccharides are composed of a single type of monosaccharide unit, whereas heteropolysaccharides contain two or more types of monosaccharides. The term **oligosaccharide** is used to denote polysaccharides with a small number of monosaccharides (usually fewer than ten). Glucose and its derivatives are the most common monomeric units; however, other monosaccharides, including fructose, galactose, and their derivatives, are found in natural polysaccharides. The polysaccharides differ from proteins in that they occur in variable sizes. They are mixtures of polymers with varying lengths; hence, they have differing molecular weights. Proteins, composed of a specific sequence and composition of amino acids, have definite molecular weights. The roles performed by the polysaccharides in energy storage and biological structure do not require molecules of a reproducible and well-defined size. In some polysaccharides, the individual strands are cross-linked by short peptides. These compounds, the peptidoglycans, are components of bacterial cell walls. In this section, we introduce the polysaccharides in terms of their biological functions, beginning with the storage polysaccharides and then continuing with those serving structural roles.

Storage Polysaccharides

Plants and animals store the energy molecule, glucose, in **starch** and **glycogen,** respectively. These polysaccharides are stored in the cell in cytoplasmic packages called granules (Figure 8.18). Starch is present in the chloroplasts of plant cells, where it is produced by photosynthetic energy (see Chapter 17). Starch is especially abundant in potatoes, corn, and wheat. Glycogen granules are present primarily in liver and muscle cells of animals. Because of the numerous hydroxyl



(a) (b)

FIGURE 8.18

Cellular segregation of storage polysaccharides in cytoplasmic granules shown in electron micrographs: (a) starch granules *(green)* in plant cell chloroplasts and (b) glycogen granules *(pink)* in liver cells.

Exhibit B

The Polysaccharides
Volume 1

Edited by

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and it is obvious that inadvertent degradation by these procedures should be kept to a minimum. In some cases, however, the very possibility of degradation may not be recognized until a vulnerable chemical linkage has been identified. It may be noted in this context that preconceptions as to purity or homogeneity may render the investigator blind to the possibility that structurally disparate regions in a natural macromolecule are indeed covalently linked. An excellent example is provided by early studies on glycosaminoglycans such as heparin and chondroitin sulfate (see Chapter 5, Volume III) which occur naturally as proteoglycans in which the glycan is covalently linked to protein through a base-sensitive O-glycosidic bond. The isolation of "pure" polysaccharide involving removal of protein by enzymatic digestion followed by extraction with dilute alkali then becomes a self-fulfilling prophecy. It may be no simple matter to distinguish between macromolecules in which segments are joined by chemically sensitive covalent bonds and those in which there are strong noncovalent interactions, and thus the severity of conditions required for dissociation may cause chemical modification so that reassociation does not occur readily. Such degradation during isolation may result in heterogeneity where none existed formerly.

II. Concepts of Homogeneity and Heterogeneity (*1*)

Many proteins, which are synthesized under direct genetic control, are *monodisperse*; i.e., all molecules, isotopic variations apart, are identical in structure and molecular weight. However, few polysaccharides, if any, are synthesized in this manner, and, even for those that are chemically and physically homogeneous, variations occur from molecule to molecule. If these variations are continuous in respect of all parameters, such as molecular size, proportions of sugar constituents, and particular linkage types, separation into discrete molecular species is impossible and the material is said to be *polydisperse*. Macromolecules that show discontinuities in molecular size but not in chemical composition are physically heterogeneous but chemically homogeneous and may be termed *polymolecular*. Chemically heterogeneous macromolecules show discontinuities in one or more of proportions of sugar constituents, linkage types, or degrees of branching. It is possible to envisage a situation in which there are mixtures of two or more monodisperse materials, but such a *paucidisperse* preparation is unlikely to be encountered other than with proteins. Gibbons (*2*) has also coined the term *heterodisperse* to denote the presence of two or more polydisperse populations of molecules. The first problems are, of course, to separate mixtures of entirely different structural types, but for certain mixtures, especially of neutral polysaccharides, there may be few distinguishing features on which to base a fractionation procedure. In addition, mixtures of polysac-

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Heterogeneity (*1*)

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charides are frequently encountered in which relatively small differences in structure may result in substantial differences in properties. For example, amylose and amylopectin, the linear and branched components of starch, respectively both consist of at least 95% of 4-linked α-D-glucopyranose residues. It is necessary, therefore, to examine ways in which polysaccharide molecules may vary in size and structure.

For homopolysaccharides of uniform linkage type, the only possible variations are of molecular size such that different molecules are *polymer homologs*, among which there may be continuous variations (polydisperse or polymolecular) or discontinuous variations with consequent physical heterogeneity. When one or more additional structural features are present in a polysaccharide, variations in structure may result from different relative proportions and/or different distributions of those features. The following examples illustrate some additional types of structural feature:

1. Linear homopolysaccharides with a second linkage type are exemplified by pullulan (**1**), an α-D-glucan, in which 4- and 6-linked residues are present in the ratio of 2:1 in a highly regular sequence (*3*). A somewhat less regular arrangement is encountered in cereal α-D-glucans (**2**), in which 3-linked residues are usually separated by two or three 4-linked residues and are only rarely in adjacent positions (*4*).

2. Linear heteropolysaccharides with two sugar constituents are of several types. Hyaluronic acid (**3**) contains a strictly alternating sequence of 4-linked β-D-glucuronic acid and 3-linked 2-acetamido-2-deoxy-β-D-glucopyranose residues. It may be noted that other polysaccharides of this general type, including other glycosaminoglycans (see Chapter 5, Volume III), are based on similar alternating sequences in which partial modification of residues of one or both types has masked the repeating unit. The 4-linked β-D-hexopyranose residues of both types in plant glucomannans (**4**) (see Chapter 3, Volume II) are arranged without apparent regularity. Alginic acid (**5**)

-[→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1-1$_n$-

1

→3)-β-D-Glc*p*-(1-[→4)-β-D-Glc*p*-(1-]$_2$→3)-β-D-Glc*p*-(1-[→4)-β-D-Glc*p*-(1-]$_3$-

2

→4)-β-D-Glc*p*-(1-[→4)-β-D-Man*p*-(1-1$_n$-

3, where $n = 0$ (infrequently), 1–6

-[→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1-]$_n$-

4

-]→4)-β-D-Man*p*A-(1-]$_x$-[→4)-L-Gul*p*A-(1-]$_y$→4)-β-D-Man*p*A-(1→4)-α-L-Gul*p*A-(1-

5

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Exhibit C

A New General Method for the Assessment of the Molecular-Weight Distribution of Polydisperse Preparations

ITS APPLICATION TO AN INTESTINAL EPITHELIAL GLYCOPROTEIN AND TWO DEXTRAN SAMPLES, AND COMPARISON WITH A MONODISPERSE GLYCOPROTEIN

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(*Received* 14 *May* 1973)

A specimen of intestinal glycoprotein isolated from the pig and two samples of dextran, all of which are polydisperse (that is, the preparations may be regarded as consisting of a continuous distribution of molecular weights), have been examined in the ultracentrifuge under meniscus-depletion conditions at equilibrium. They are compared with each other and with a glycoprotein from *Cysticercus tenuicollis* cyst fluid which is almost monodisperse. The quantity $c^{-\frac{1}{2}}$ (c = concentration) is plotted against ξ (the reduced radius); this plot is linear when the molecular-weight distribution approximates to the 'most probable', i.e. when $M_n:M_w:M_z:M_{(z+1)}$ ..... is as 1:2:3:4: etc. The use of this plot, and related procedures, to evaluate qualitatively and semi-quantitatively molecular-weight distribution functions where they can be realistically approximated to Schulz distributions is discussed. The theoretical basis is given in an Appendix.

In principle the radial macromolecular-concentration distribution at equilibrium in the ultracentrifuge contains all the information necessary to characterize the molecular-weight distribution function and methods have been described for doing this (Billick *et al.*, 1967; Provencher, 1967; Sundelöf, 1968; Scholte, 1968, 1969; Magar, 1970; Wiff & Gehatia, 1972; Williams, 1972). These techniques require a good deal of experimental and computational effort; the result, moreover, is often not unique. It would be valuable to have a simpler method for assessing approximately the broadness of a molecular-weight distribution of a biochemical preparation known to be homogeneous but polydisperse in the sense described by Gibbons (1963). This condition does not commonly arise with proteins, but polysaccharides, many glycoprotein and some nucleic acid preparations do present this obstacle to proper biophysical characterization. In these cases the extrapolation necessary to obtain the required parameters at the cell base is difficult, as the usual logarithmic plot may be quite curved and a function more nearly linear is desirable.

This investigation originated from the theoretical considerations given in the Appendix. With symbols as defined in Table 1, for the so-called 'most-probable' distribution for which the ratios of successive molecular-weight averages are in arithmetic progression, a plot of $c^{-\frac{1}{2}}$ against ξ will be linear provided that c, all its derivatives with respect to ξ and the two integrals $\int c\,d\xi$ and $\int(\int_{\xi}^{\infty} c\,d\xi)\,d\xi$ have values that are negligibly small at the meniscus compared with the value at the base of the cell. This in effect is the widely used meniscus-depletion condition (Yphantis, 1964) where $c_b > 10^3 \times c_m$. However, the relevant function $c = 2q^2/(q\xi+k)^3$ gives, for the second integral above, the values $1/k$ and $1/(q+k)$ at the cell base and the meniscus respectively, and for the latter to be negligible compared with the former, q must be much greater than k. This imposes a very much more stringent restriction on the ratio of c_b/c_m than does the Yphantis (1964) condition, for if $c_b/c_m = 10^3$, the ratio of the two quantities $1/k$ and $1/(q+k)$ is 10. Nevertheless, even with values of this order of magnitude, linearity is very good for materials having molecular-weight averages close to the most-probable distribution, the only serious discrepancy being that of M_n/M_w, which becomes 0.54 in place of the ideal 0.5.

Two samples of dextran that are considered to have continuous molecular-weight distributions close to the most-probable one have been examined to explore the usefulness of this proposal. The results are compared with those obtained with an essentially monodisperse glycoprotein and with those found for an intestinal glycoprotein preparation in which the distribution is broader than the most-probable one.

Experimental

Materials

Glycoproteins. (i) *Tenuicollis* glycoprotein. This material was isolated from the cyst fluid of the tapeworm *Cysticercus tenuicollis* in the goat (Dixon *et al.*, 1973). (ii) Porcine intestinal glycoprotein. This material was isolated by squeezing the contents of 1m lengths of the intestinal tracts (duodenum and

Table 1. *Definition of symbols used*

c = concentration
r = radius of rotation
$\bar{v}$ = partial specific volume of macromolecular species
ρ = solvent density
ω = angular velocity
R = gas constant
T = temperature (°K)
q, k = empirical constants as defined in the Appendix [eqn. (9)]
p, h = empirical constants as defined in the Appendix [eqn. (10)]
$\alpha = 1/(h+2)$

M = molecular weight, the subscripts n, w, z, $(z+n)$ indicating the number, weight, z, or $(z+n)$ average respectively, n being any positive integer. The subscripts m, b, and o appended to the variables c, r and ξ, and to derivatives of these quantities, imply the value of the subscripted variable at the meniscus, at the base of the cell and before the commencement of the equilibrium experiment respectively. Two derived quantities are also used: the experimental constant $\lambda = [(1-\bar{v}\rho)(r_b^2-r_m^2)\omega^2]/2RT$ and the reduced radius of rotation $\xi = (r_b^2-r^2)/(r_b^2-r_m^2)$.

Table 2. *Ultracentrifugal analysis of glycoproteins and dextrans by the method of Chervenka* (1970)

The initial concentration was that introduced into the ultracentrifuge cell before formation of the boundary. M_w(app.) was calculated as described in the text. All runs were carried out at 293°K.

	Initial concn. (mg/ml)	Mean speed of centrifugation (rev./min)	Time (h)	$\bar{v}$	M_w(app.)
C. tenuicollis glycoprotein	0.25	18300	43	0.74	96800
Pig intestinal glycoprotein	3.0	5500	100	0.66	3.31×10^5
	0.6	5500	100	0.66	5.34×10^5
	0.3	5500	100	0.66	8.54×10^5
Dextran 1	0.5	16600	76	0.613	35200
Dextran 2	0.25	18300	100	0.613	66900

jejunum) of pigs into saline at 4°C, then removing the bulk of the food particles by filtration through muslin. It was purified by digestion with crystalline pepsin (0.05mg/ml) at pH3.5 and 37°C for 48h, followed by extraction with 90% (w/v) phenol as described by Morgan & King (1943). The aqueous extract from the phenol treatment was dialysed against ten changes of water at 4°C and freeze-dried. The solid was then fractionated by gel filtration on a column (2.5cm×100cm) of Sephadex G-200 in 0.05M-sodium acetate buffer, pH6.5, containing 0.02% (w/v) NaN_3. This yielded two major components, an excluded and an included fraction; both fractions were subsequently recovered by dialysis against ten changes of water at 4°C followed by freeze-drying. The higher-molecular-weight material (excluded fraction) was used in the work reported here. (iii) Dextran. Two samples were used; dextran 1 was a partially acid-hydrolysed unfractionated sample and dextran 2 a 'clinical' dextran with a stated molecular weight of 60000–90000.

Solvents. (i) 0.01M-Sodium barbiturate buffer, pH7.0, containing 0.1M-NaCl; (ii) 0.15M-NaCl.

Methods

Preparations of samples. Solutions of *C. tenuicollis* glycoprotein, 0.02% in solvent (i), of porcine intestinal glycoprotein, 0.3% in solvent (i), and of the two dextrans, 0.05 and 0.025% respectively in solvent (ii), were prepared. Each solution was dialysed against 10vol. of the appropriate solvent for 24h at 4°C and subsequently centrifuged at 20000rev./min (r_{av}. 2.5cm) for 16h to eliminate any gelatinous aggregates or undissolved material. Dilutions of 1:5 and 1:10 of the porcine intestinal glycoprotein solutions were also made.

Analytical ultracentrifugal examinations. All six solutions were examined in the synthetic-boundary cell of the Spinco model E ultracentrifuge as described by Chervenka (1970) by using 0.15ml of solution plus 0.02ml of silicone oil and 0.4ml of solvent plus 0.04ml of silicone oil. Conditions of each run are given in Table 2. Equilibrium was checked before termination of each run by the comparison of two plates exposed at intervals of 8 or 12h. The cell base was located after running speed was attained by making an exposure using schlieren optics with the

phase-plate angle at 90°. Exposures using light of wavelength 541.1nm were made immediately after running speed was reached as well as at the end of the run. Exposures with white light were also made at these times and the comparison of the position of the zero-order fringe at the meniscus gave an additional check that this identifiable fringe had not moved perceptibly at the meniscus, i.e. that the meniscus concentration was actually zero.

Solvent densities were measured in a 50ml pyknometer. Partial specific volumes were calculated from the sugar and amino acid composition; values for the appropriate residues were taken from Cohn & Edsall (1943) and Gibbons (1972). Plates were

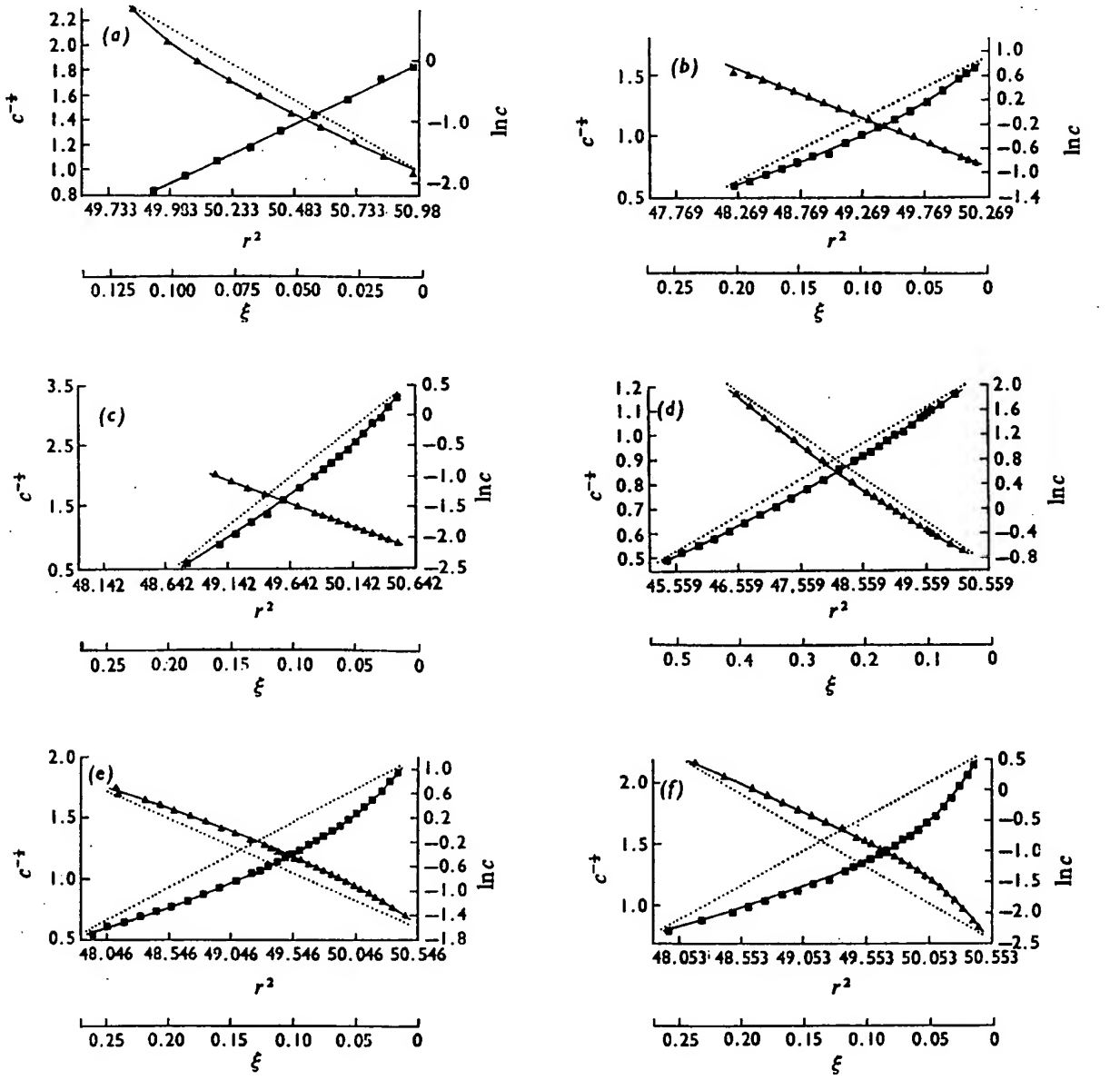

Fig. 1. *Equilibrium distributions in the ultracentrifuge under meniscus-depletion conditions*

The method of Chervenka (1970) was used; for conditions see Table 2. Concentrations, c, are in arbitrary units, r is in cm and $\xi = (r_b^2 - r^2)/(r_b^2 - r_m^2)$. ■, ln c against r^2; ▲, $c^{-\frac{1}{2}}$ against ξ. The broken line indicates linearity where the observed plot is not linear. (*a*) *C. tenucollis* glycoprotein, 0.02%; (*b*) dextran 1, 0.05%; (*c*) dextran 2, 0.025%; (*d*), (*e*) and (*f*) pig intestinal glycoprotein examined at 0.3%, 0.06% and 0.03% respectively.

measured with a tool-maker's micro-comparator (Precision Grinding Instrument Co., Mitcham Junction, Surrey, U.K.) and aligned by using the air fringes. Measurements along the r axis were made at intervals of 0.02cm. The data were processed by using an Olivetti P 101 calculator. The values of M_w (app.) (not corrected for non-ideal behaviour, Table 2) were calculated as $c_b/\lambda c_o$ [see Appendix, eqn. (2)]; c_o was calculated as $\int_0^1 c\,d\xi$ both from the initial and final green-light exposures, and c_b was calculated by extrapolation of either the plot of $\ln c$ against r^2 or of $c^{-\frac{1}{2}}$ against ξ, whichever was most linear.

Results and Discussion

The 'most-probable' distribution of molecular weights is a Schulz distribution with $h = 1$ (Schulz, 1944) and is of peculiar significance, as it is the molecular-weight distribution resulting from the random break-up of an indefinitely large molecule (Pathria & Nanda, 1959) or from macromolecule synthesis by random chain-lengthening (Tanford, 1961). For material having a continuous molecular-weight distribution, with one maximum and two points of inflexion only, it is often valuable to know whether the distribution is broader or narrower than the most-probable one. Under meniscus-depletion conditions, the amount of and direction of curvature in a plot of $c^{-\frac{1}{2}}$ against ξ will immediately give information about this point with the minimum of extra work. The two dextran preparations, although giving markedly curved plots of $\ln c$ against c/r^2, gave plots of $c^{-\frac{1}{2}}$ against ξ that were highly linear (Figs. 1*b* and 1*c*) and the correlation coefficients for samples 1 and 2 were 0.9996 and 0.9994 respectively. Extrapolation to $\xi = 0$ can be made with considerable confidence and from the measured values of q and k any molecular-weight average may be deduced. On the other hand, the epithelial glycoprotein examined gave marked curvature in the plots of $c^{-\frac{1}{2}}$ versus ξ (Figs. 1*e* and 1*f*) except at the highest concentration examined (Fig. 1*d*); this indicates that these materials have a wider distribution of molecular weights than that expected from random synthesis or breakdown, a conclusion already reached by Creeth & Knight (1968) for a similar, human, epithelial glycoprotein. Further, the curve cannot be made satisfactorily linear by selecting a different exponent between $-\frac{1}{3}$ and $-\frac{1}{2}$ (Fig. 2), which implies that a Schulz distribution is inadequate as a representation of the molecular-weight distribution of this glycoprotein. Creeth & Denborough (1970) have shown that their sample of epithelial glycoprotein is polydisperse with respect to partial specific volume as well, a conclusion that most probably applies to the similar glycoprotein studied here. Thus the distribution function is being examined of not M but $M(1-\bar{v}\rho)$; until the distribution in $\bar{v}$ can be further elucidated this point should always be remembered. In addition the effect of non-ideal behaviour is evident for the epithelial glycoprotein; this reveals itself as an apparent narrowing of the molecular-weight distribution as concentration is increased (Figs. 1*d*, 1*e* and 1*f*). At the lowest concentrations, where ideal conditions are approached, a distribution wider than the most-probable one is apparent. Concentration effects must therefore be taken into account in the assessment of molecular-weight distributions, just as for the assessment of monodispersity with a plot $\ln c$ against r^2. A condition of 'pseudo-most-probable distribution', entirely analogous to the spurious appearance of monodispersity in plots of $\ln c/r^2$, can arise from non-ideal behaviour (Fig. 1*d*). In view of the relatively low molecular weights of the two dextran samples and of the low concentrations at which they were examined, the disturbances due to the second virial coefficient should be small with this polysaccharide (Berry & Casassa, 1970; Senti *et al.*, 1955; Jeans *et al.*, 1954). The *C. tenuicollis* glycoprotein is included (Fig. 1*a*) in the data for comparative purposes; it appears to be a globular protein that is very nearly monodisperse.

The assumption that $\int (\int_\xi^\infty c\,d\xi)\,d\xi$ has a negligible value at $\xi = 1$ is not justified at the angular velocities used here; nevertheless it appears to cause little disturbance to the linearity of the plots where the materials have a molecular-weight distribution close to the most-probable one. It may be calculated that for dextran 1 successive molecular-weight ratios are 0.566, 0.678, 0.75, 0.8, etc. and for dextran 2 are 0.54,

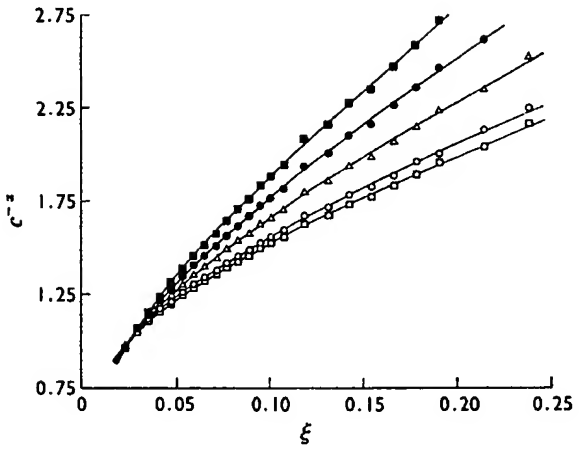

Fig. 2. *Curves relating $c^{-\alpha}$ to ξ for different values of the exponent α for pig intestinal glycoprotein* (0.03%)

Reference to the Appendix, second section, shows that for Schulz distributions wider than the most-probable distribution this will become linear for some value of α between $\frac{1}{3}$ and $\frac{1}{2}$. □, $\alpha = 0.03$; ○, $\alpha = 0.35$; △, $\alpha = 0.4$; ●, $\alpha = 0.45$; ■, $\alpha = 0.5$.

0.671, 0.75, 0.8, etc. if the graphs are assumed to be strictly linear. Where narrower distributions are encountered, the exponent α (see the Appendix) becomes less than $\frac{1}{3}$ and such distributions may more readily be characterized by linearity of the appropriate plot, because the smaller the exponent the less serious is the assumption that $\int (\int_{\xi}^{\infty} c\,d\xi)d\xi$ is 0 at $\xi = 1$. For example, with a Schulz distribution having $h = 2$, $c^{-\frac{1}{2}}$ is linear with ξ to a high degree of accuracy if $c_m < 10^{-3} \times c_b$.

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APPENDIX

Derivation of an Approximate Function Relating c and ξ for Schulz Molecular-Weight Distributions in the Meniscus-Depletion Condition in the Equilibrium Ultracentrifuge

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(*Received* 14 *May* 1973)

Most-Probable Case

With the notation from Table 1 of the main paper (Gibbons *et al.*, 1973), successive molecular-weight averages may be written (Fujita, 1962):

$$M_n = \frac{c_o/\lambda}{\left[\int_0^1 \left(\int_{\xi}^{\infty} c\,d\xi\right) d\xi\right]} \quad (1)$$

$$M_w = \frac{1}{\lambda c_o}(c_b - c_m) \quad (2)$$

$$M_z = \frac{1}{\lambda^2 c_o M_w}\left[\left(\frac{dc}{d\xi}\right)_m - \left(\frac{dc}{d\xi}\right)_b\right] \quad (3)$$

and generally:

$$M_{(z+n)} = \frac{1}{\lambda^{(n+2)} c_o M_w M_z \ldots M_{(z+n-1)}} \times \left[\left(\frac{d^{(n+1)}c}{d\xi^{(n+1)}}\right)_m - \left(\frac{d^{(n+1)}c}{d\xi^{(n+1)}}\right)_b\right] \quad (4)$$

if n is an even integer; if it is odd then the term within the square brackets becomes

$$\left[\left(\frac{d^{(n+1)}c}{d\xi^{(n+1)}}\right)_b - \left(\frac{d^{(n+1)}c}{d\xi^{(n+1)}}\right)_m\right]$$

The ratios of successive molecular-weight averages simplify to

$$\frac{M_n}{M_w} = \frac{c_o^2}{\left[\int_0^1 \left(\int_{\xi}^{\infty} c\,d\xi\right) d\right](c_b - c_m)} \quad (5)$$

Exhibit D

The Polysaccharides
Volume 3

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1985

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With the possible exception of hyaluronate, all connective tissue polysaccharides are covalently attached to protein. The protein part of the macromolecule is also substituted with N-linked or O-linked oligosaccharides (Fig. 1) in much the same way as regular glycoproteins or mucins. Hence, proteoglycans may be viewed as specialized glycoproteins characterized by the presence of polysaccharide side chains. The latter can be extensions of N-linked oligosaccharides, as in the case of corneal keratan sulfate (Fig. 1e), can be extensions of O-linked, mucin-type saccharides such as the cartilage keratan sulfate (Fig. 1a), or can be built onto a special linkage region (**1**) as in

-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Xyl*p*-(1→3)-L-Ser

1

the case of chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin *(6)*.

The physicochemical properties of proteoglycans are determined, by and large, by the nature of the polysaccharide side chains. The hydrodynamic features of these chains are described elsewhere in this treatise (Chapter 5, Volume 1), where, for example, their capacity to occupy large hydrated domains is pointed out. When many such chains are bound to a common core protein, the extended structures of these macromolecules may encompass solvent volumes 50 times or more that of their dry mass. In addition, a proteoglycan like that of cartilage may comprise as many as 10^4 negatively charged groups per molecule. These features enable the proteoglycans to exert an outstanding resistance to compressive forces. The smaller proteoglycans may convey other properties, again via their polysaccharide side chains, because these may participate in specific intermolecular associations (see Section IV).

III. Glycosaminoglycans

A. General

1. Classification

The classification of glycosaminoglycans is based on convenient but arbitrary criteria. We generally divide them into the following groups: hyaluronate, chondroitin 4- or 6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin. However, there is considerable variability (e.g., polydispersity in molecular weight and heterogeneity in sugar composition). Dermatan sulfates vary with regard to uronic acid composition (GlcA, IdoA, and IdoA-OSO_3), chondroitin sulfates vary in the proportion of 4- to 6-sulfa-

Exhibit E

CHEMISTRY AND BIOLOGY OF HYALURONAN

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Chemistry and Biology of Hyaluronan
H.G. Garg and C.A. Hales (editors)

Chapter 3

Methods for Determination of Hyaluronan Molecular Weight

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I. Introduction

Hyaluronan samples are not generally monodisperse in molecular weight. When we characterize the molecular weight, we either determine an average molecular weight, or we characterize the distribution of molecular weights present. The type of average measured depends on the method used. A method such as light scattering (LS), the signal for which depends on the molecular weight of each species present, determines a weight-average molecular weight. A method such as osmometry or end group analysis, for which the number of molecules of each size is determined, gives a number-average molecular weight. The ratio of the weight-average to the number-average molecular weights is the polydispersity index, which is equal to one for a monodisperse sample, but greater than one for a polydisperse sample. Consider a sample containing just two different molecular weights (m_i) of a polymer, 1×10^6 and 2×10^6. If the two species are present in equal weights (w_i), 1 g each, then the weight average molecular weight is

$$\overline{M}_w = \frac{\sum w_i m_i}{\sum w_i} = \frac{1(1 \times 10^6) + 1(2 \times 10^6)}{1+1} = \frac{(1 \times 10^6) + (2 \times 10^6)}{2}$$

$$= 1.5 \times 10^6 \qquad (1)$$

The number average is calculated by recognizing that the number of moles (n_i) of a species present is equal to its weight divided by its molecular weight

(g/(g/mol) = mol). The previously described sample contains 1×10^{-6} mole of the species with a molecular weight of 1×10^6, and 0.5×10^{-6} mole of the species with a molecular weight of 2×10^6. The number average molecular weight is thus

$$\overline{M}_n = \frac{\sum n_i m_i}{\sum n_i} = \frac{(1 \times 10^{-6})(1 \times 10^6) + (0.5 \times 10^{-6})(2 \times 10^6)}{(1 \times 10^{-6}) + (0.5 \times 10^{-6})}$$

$$= \frac{1+1}{1.5 \times 10^{-6}} = 1.33 \times 10^6 \tag{2}$$

and the polydispersity is $M_w/M_n = 1.13$.

We shall discuss in detail only three types of methods for the determination of hyaluronan molecular weight, because these are the most widely used methods today. Viscometry allows the routine determination of viscosity-average (close to weight-average) molecular weight for hyaluronan over a wide range of molecular weights. Electrophoretic techniques may be used for characterization of hyaluronan molecular weight distributions (MWDs) ranging from oligosaccharides to polymers with molecular weights up to about 6×10^6. LS gives a weight-average molecular weight and when used in conjunction with separation by size exclusion chromatography (SEC) the complete MWD may be determined.

II. Viscometry

A. Theory

Measurement of the viscosity of a solution containing hyaluronan allows the determination of the polymer viscosity-average (close to the weight-average) molecular weight. The theoretical basis for this effect is conceptually simple (1). The Stokes–Einstein relation for the specific viscosity, η_{sp}, of a dilute suspension of n spherical particles per unit volume of suspension, each with volume V, is

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} = 2.5nV \tag{3}$$

where η and η_0 are the respective viscosities of the suspension and pure fluid. The product, nV, is the volume fraction (sometimes denoted by φ) of the suspension occupied by the particles. Thus this equation states that the extent to which the suspension viscosity is greater than the pure fluid is determined by the fraction of the volume which is filled with particles.

For a polymer solution, the corresponding relation is

$$\eta_{sp} = 2.5\left(\frac{cN_A}{M}\right)\nu_h = 2.5c\left(\frac{N_A \nu_h}{M}\right) = 2.5\, cV_s = c[\eta] \tag{4}$$

where c is the polymer concentration in g/cm^3, N_A Avagadro's number, M the polymer molecular weight, ν_h the hydrodynamic volume in cm^3 of a single

unexplained shadow peaks complicated interpretation of the profile. Kakehi et al. (59) and Kinoshita et al. (60) employed polysiloxane-coated capillaries (to eliminate electro-osmotic flow) and a polyethylene glycol entangled polymer matrix in a Tris–borate buffer to obtain excellent separation of hyaluronan oligosaccharides up to 100 disaccharides in length. Detection was by absorbance at 200 nm. Such separations have the potential to replace PAGE for hyaluronan oligosaccharide and fragment analysis, but it is important to note that quantitative correlation of the electropherograms with independent methods for analysis of MWD has not yet been established.

D. Agarose Gel Electrophoresis of High Molecular Weight Hyaluronan

Lee and Cowman (61) adapted methods used in the electrophoretic separation of high molecular weight nucleic acids for the separation of high molecular weight hyaluronan. They proposed the use of agarose gel at 0.5% in a continuous Tris–acetate–EDTA buffer for the separation of hyaluronan (Fig. 6). Sample loads of approximately 4–7 μg were required for polydisperse samples and the separated pattern was visualized by staining with the dye Stains-All (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine). For hyaluronan standards of known average molecular weight, the electrophoretic mobility was found to be approximately linearly related to the logarithm of the hyaluronan molecular weight over the range of $0.2 \times 10^6 - 6 \times 10^6$. Larger molecules may be separable by this method, but no suitable standards have been available. The method was shown to be useable preparatively, but yields are low and some degradation occurs during extraction of the hyaluronan from the gel. Impure hyaluronan samples containing high levels of contaminating protein (e.g., synovial fluid) were found to require predigestion with a proteolytic enzyme. Contaminating sulfated glycosaminoglycans were readily identifiable by their faster mobility and different color upon staining. The hyaluronan could be transferred from the gel by semi-dry electroblotting onto positively charged nylon. Hyaluronan could be subsequently detected by staining the membrane with alcian blue, or it could be specifically stained using biotin-labeled HA binding protein and streptavidin-gold followed by silver staining for sensitive visualization.

AGE proved to be a facile method for the determination of the MWD of hyaluronan. It was applied to the characterization of low molecular weight hyaluronan samples implicated in the induction of inflammatory gene expression in macrophages (62,63). It was used to demonstrate the degradation of hyaluronan by peroxynitrite, which may be generated during inflammation by the reaction of nitric oxide with superoxide anion (31). The pattern of degradation in the presence of different scavengers was similar to that caused by hydroxyl radicals. The average molecular weight determined by electrophoresis for hyaluronan as a function of degradation by peroxynitrite was in good agreement with the results obtained by viscometric analysis.

Recently an improved blotting and detection procedure was developed by Armstrong and Bell (64). A number of nylon-based membranes were tested for

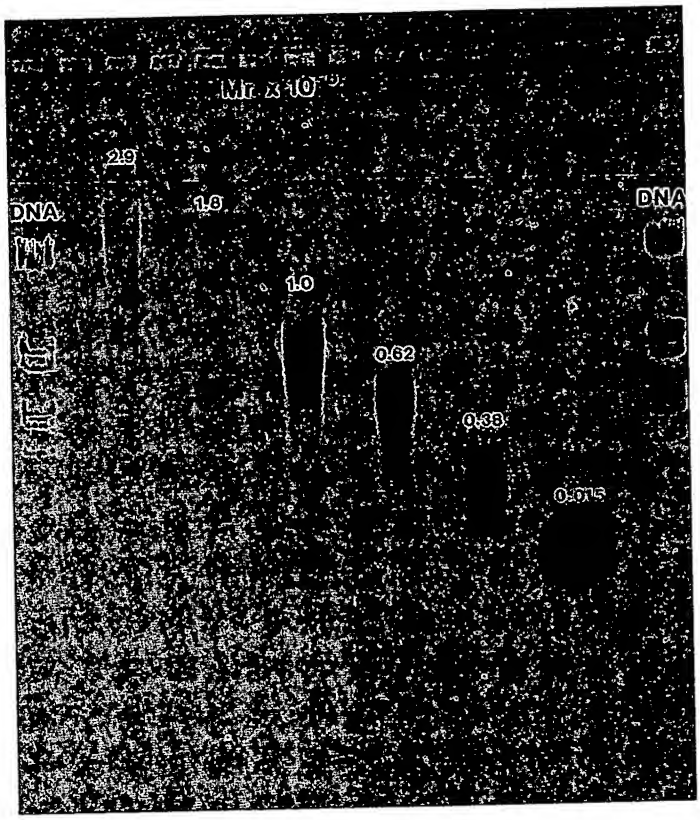

Figure 6 Agarose gel electrophoresis of low polydispersity hyaluronan samples obtained by electrophoretic fractionation and subsequent recovery from the gel. Low molecular weight hyaluronan migrates more rapidly than high molecular weight hyaluronan. From Ref. 61.

hyaluronan binding, and the two best were found to be Gene Screen plus and Hybond-N^+. Hyaluronan was transferred from the electrophoretic gel by capillary blotting, and the separation pattern detected by binding of ^{125}I-labeled HA binding protein and autoradiography. The authors employ their procedure to analyze hyaluronan from solid tissues after protease digestion in the presence of desferoxamine. Although the exact molecular weight was impossible to determine because it was higher than the standards employed, the fraction of very high molecular weight hyaluronan ($>4 \times 10^6$) was approximately 58% for hyaluronan from tissues as diverse as skin, skeletal muscle, heart, lung, small intestine, and large intestine. This surprising result indicated that normal isolation procedures generally degrade hyaluronan and that in the tissue the average molecular weight is probably on the order of 6×10^6, as it was found to be in human knee joint synovial fluid and owl monkey eye vitreous (61).

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reous (61).

Slightly higher agarose concentrations were used by Pummill and DeAngelis (65) to optimize the separation of hyaluronan in the 0.2×10^6–1×10^6 range and study the molecular weight of hyaluronan produced by single amino acid mutated forms of a vertebrate hyaluronan synthase. Radio-labeled hyaluronan was separated on 1.35% agarose gel and detected by fluorography. The mutated forms were shown to produce hyaluronan of larger or smaller size, determined by the nature of the single site mutation.

A major handicap in the use of AGE to determine hyaluronan MWD has been the difficulty in obtaining suitable molecular weight standards. Recently, nearly monodisperse hyaluronan standards have been produced by Hyalose LLC. The pmHAS enzyme, the HA synthase from the Gram-negative bacterium *Pasteurella multocida*, catalyzes the synthesis of HA polymer utilizing monosaccharides from UDP-sugar precursors. The recombinant pmHAS will also elongate exogenously supplied HA oligosaccharide acceptors *in vitro* (66). HA oligosaccharides substantially boost the overall incorporation rate in comparison to *de novo* synthesis of HA polymer chains because chain initiation is slower than chain elongation. The chemoenzymatic synthesis of HA polymers of any desired molecular weight ($\sim 5 \times 10^3$ to $\sim 1.5 \times 10^6$) with very narrow size distributions using pmHAS has been developed ('selectHA', Jing and DeAngelis, in preparation). HA polymers of a desired size are produced by controlling the reaction stoichiometry (i.e., ratio of UDP-sugar precursors and acceptor molecules). The total amount of precursors determines the final mass of HA polymer that can be synthesized. If a small number of acceptor molecules (e.g., HA tetrasaccharide) are present in the reaction mixture, then a few long chains will be made. Conversely, if a large number of acceptor molecules are present, then many short chains will result. The polymerization process is synchronized in the presence of acceptor (i.e., bypassing slow *de novo* initiation step) thus all polymer products are very similar. In contrast, reactions without acceptor produce HA polymers with a wider size distribution. Each specific size class of selectHA had a polydispersity value in the range of 1.01–1.2 (1 is the ideal monodisperse size distribution) as assessed by SEC/multi-angle laser LS analysis. The selectHA preparations migrate on electrophoretic gels (agarose or polyacrylamide) as very tight bands facilitating their use as size standards (Fig. 7). Furthermore, these HA preparations should be of great utility for elucidating the relationship between HA size and its biological activities.

IV. Light Scattering and Size Exclusion Chromatography

Fundamental properties of hyaluronan, such as viscoelasticity and flow behavior primarily depend on the MWD, size, and conformation of the macromolecules. A primary method in estimating the molecular weight and the size of macromolecules is LS. LS and a few other methods such as osmometry, sedimentation, and mass spectrometry are absolute techniques. However, only the LS technique can be used online to a SEC system in obtaining the whole

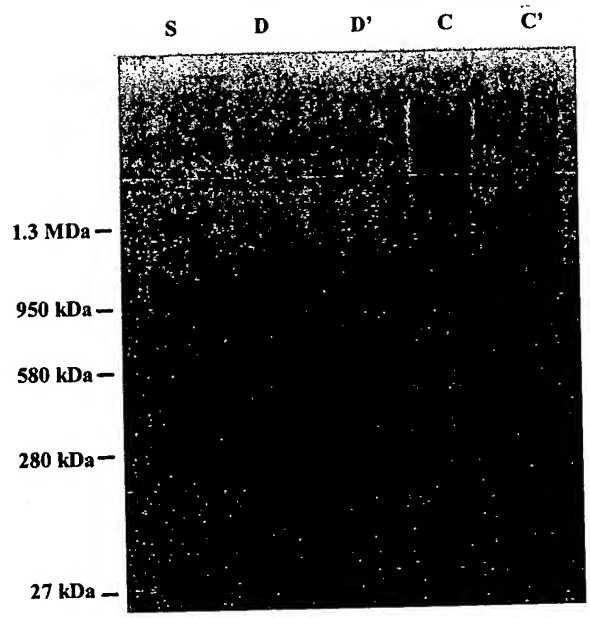

Figure 7 Agarose gel electrophoresis of nearly monodisperse hyaluronan standards and commercial hyaluronan. Gel was 0.7% agarose in Tris–acetate–EDTA (minigel format), stained with Stains-All by the method of Lee and Cowman (61). S: a mixture of 5 different monodisperse SelectHA preparations with indicated M_w determined by SEC–MALS; C and C′: commercial hyaluronan samples; D: DNA standards, Bioline Hyperladder 1, containing DNA of 10, 8, 6, 5, 4, 3, 2.5, 2, 1.5 kb; D′: DNA standards, BioRad 1 Kilobase Ruler, containing DNA of 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 kb. Figure kindly provided by P DeAngelis and W Jing.

MWD. As a consequence, LS is a fundamental method for the characterization of hyaluronan.

LS concerns the interaction of light with matter in the specific case with macromolecules in solution. The interaction of light with matter is a very complex topic. Depending on the type of scattering analyzed (elastic, quasi-elastic, Raman, etc.) different information may be obtained. For the characterization of macromolecules (molecular weight and size) only elastic and quasi-elastic LS are of interest. In an elastic LS experiment (also known as static or total intensity or Rayleigh scattering) we measure the intensity of the scattering. In this case, we assume that the scattered light has the same wavelength and polarization of the incident light. On the contrary, in a quasi-elastic LS experiment (also known as dynamic or photon correlation spectroscopy) we measure the fluctuations of the intensity of the scattering due to the Brownian movement of the macromolecules.

Following Zimm (67) the intensity of the scattering of a solution of macromolecules is related to the molecular weight M of the sample by the